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Intravirion Processing of the Human Immunodeficiency Virus Type 1 Vif Protein by the Viral Protease May Be Correlated with Vif Function

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The human immunodeficiency virus type 1 (HIV-1) Vif protein is specifically packaged into virus particles through an interaction with viral genomic RNA in which it associates with the viral nucleoprotein complex. We now demonstrate for the first time that virus-associated Vif is subject to proteolytic processing by the viral protease (Pr). Pr-dependent processing of Vif was observed both in vivo and in vitro. In vivo processing of Vif was cell type independent and evident by the appearance of a 7-kDa processing product, which was restricted to cell-free virus preparations. Processing of Vif required an active viral Pr and was sensitive to Pr inhibitors such as ritonavir. The processing site in Vif was characterized both in vivo and in vitro and mapped to Ala_{150}. Interestingly, the Vif processing site is located in a domain that is highly conserved among HIV-1, HIV-2, and simian immunodeficiency virus Vif isolates. Mutations at or near the processing site did not affect protein stability or packaging efficiency but had dramatic effects on Vif processing. In general, mutations that markedly increased or decreased the sensitivity of Vif to proteolytic processing severely impaired or completely abolished Vif function. In contrast, mutations at the same site that had little or no effect on processing efficiency also did not influence Vif function. None of the mutants affected the ability of the virus to replicate in permissive cell lines. Our data suggest that mutations in Vif that cause a profound change in the sensitivity to Pr-dependent processing also severely impaired Vif function, suggesting that intravirion processing of Vif is important for the production of infectious viruses.

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vif plays an important role in regulating virus infectivity (17, 55). The lack of a functional Vif protein results in the production of virions with reduced or abolished infectivity (17, 30, 55). This effect of Vif on virus infectivity is producer cell dependent and can vary by several orders of magnitude (2, 6, 7, 16, 17, 20, 30, 42, 55, 56). Virus replication in nonpermissive cell types such as primary T cells and macrophages as well as a small number of T-cell lines, including H9, is strictly dependent on Vif, while Vif-defective viruses can efficiently replicate in permissive hosts such as Jurkat cells. The cellular factors determining the requirement for Vif are currently not known. Results from heterokaryon analyses, which involved the fusion of restrictive with permissive cell types, suggested the presence of an inhibitory factor in restrictive cell types (34, 47). However, the identity of the proposed inhibitory factor and its mode of action remain under investigation.

Although *vif* genes are present in all lentiviruses, with the exception of equine infectious anemia virus, there is relatively little sequence conservation between different Vif variants (36). Nevertheless, HIV-1 Vif was found to be capable of functionally complementing Vif-defective HIV-2 and simian immunodeficiency virus (SIV) macaque isolates. Similarly, HIV-2 Vif was capable of complementing HIV-1 Vif defects, suggesting common functional domains and a common mode of action (41, 50).

Vif is a 23-kDa basic protein that is expressed late during infection in a Rev-dependent manner (44). Immunocytochemical analyses reveal a largely cytoplasmic localization of Vif (21, 28, 46). In particular, confocal microscopy revealed that a significant amount of Vif associates with the intermediate filament network in virus-producing cells (28) causing severe alterations of the intermediate filament structure (24, 28); however, the domain(s) in Vif responsible for this association as well as its functional significance remain to be determined. Recent reports also suggest that Vif associates with viral genomic RNA in vivo and in vitro, and deletions in the N-terminal and central regions were found to affect the ability of Vif to bind to poly(G)-conjugated agarose beads in vitro (13, 63). Aside from showing an affinity for RNA and intermediate filaments, Vif was reported to associate with cellular membranes through a mechanism involving a basic C-terminal domain in Vif (21, 22, 53). This same domain also was reported to be responsible for the interaction of Vif with the Gag precursor $Pr55^{gag}$ (8) and was found to influence multimerization of Vif in vitro and in vivo (60). Thus, despite its low degree of conservation among distant isolates, the C-terminal domain of Vif appears to be a major contact point for viral or host factors. Other domains in Vif have similarly important functions. In fact, mutational analyses of Vif demonstrated that changes in

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all regions of Vif can lead to loss of biological activity consistent with a multidomain structure of the protein (10, 29, 33, 43, 51).

Despite the severe impact of Vif mutations on virus infectivity, Vif's mechanism of action has thus far remained obscure. It is generally accepted that Vif-deficient viruses can attach to and penetrate host cells but are blocked at a postpenetration step early in the infection cycle (3, 11, 12, 41, 48, 56). Yet, comparison of virion morphology or protein composition between wild-type and Vif-defective virions has thus far been inconclusive (7, 9, 18, 25, 38, 45). Several reports have suggested that Vif affects the stability of the viral nucleoprotein complex (25, 39, 48). In particular, nucleocapsid (NC) and reverse transcriptase were found to be less stably associated with viral cores in the absence of Vif (39). Nevertheless, Vif is generally believed to function within the virus-producing cell. This assumption is largely based on the observation that relatively small amounts of Vif seem to be packaged, with estimates ranging from less than 1 to 100 molecules of Vif per virion (10, 14, 18, 32).

Based on this information, we recently performed an in-depth biochemical analysis of Vif in purified virions from permissive and restrictive host cells to investigate the specificity of Vif incorporation into virions (29). Detergent extraction of purified virions verified earlier observations demonstrating the association of Vif with nucleoprotein complexes (28, 29, 32). Our own quantitative analyses suggested that approximately 10 to 15% of total Vif protein is packaged into virions (29). This efficiency is comparable to the 5% packaging efficiency reported for the HIV-1 Env protein (58). Interestingly, HIV-1 variants carrying mutations in the NC zinc finger domains abolished Vif packaging. In addition, a helper virus carrying a deletion of the RNA-packaging signal was significantly impaired in packaging of Vif. Finally, deletion of a putative RNA-binding motif between residues 75 and 114 in Vif abolished its packaging into virions. Virion-associated Vif was resistant to detergent extraction and copurified with components of the viral nucleoprotein complex and functional reverse transcription complexes (29). All of these data suggested that packaging of Vif is specific and mediated through an interaction with the viral genomic RNA and possibly NC (29).

In the present study, we investigated the stability of Vif in HIV-1 virions. Time course analyses of metabolically labeled virus preparations demonstrated that virus-associated Vif is gradually degraded following virus release. In fact, we found that Vif is subject to intravirion processing by the HIV-1 protease (Pr). Intravirion processing of Vif was observed in virus derived from permissive HeLa and Jurkat cells as well as restrictive H9 cells. Intravirion processing of Vif was not observed when a viral variant carrying an active-site mutation in the protease gene was tested. In addition, intravirion processing of Vif was sensitive to inhibitors of the viral protease. Interestingly, no Pr-dependent intracellular processing of Vif was observed. This suggests that Vif processing is restricted to cell-free virions. Microsequencing of in vitro-processed Vif protein revealed that processing occurred in a highly conserved domain of the protein. Processing site mutations were found to have various effects on the sensitivity of Vif to Pr-dependent processing. Importantly, mutations in Vif that affected Pr-dependent processing also affected the ability of Vif to regulate viral infectivity, suggesting that intravirion processing of Vif is functionally relevant and pointing to a function of Vif as a component of the viral core.

TABLE 1. Characterization of Vif processing site mutants

Processing site	Mutation(s)	Sensitivity to processing[a]	Replication in H9 cells[b]
Vif wild type	NA[c]	Normal	Normal
Vif-RKS	$A_{149}R$, $L_{150}K$, $A_{151}S$	Resistant	No replication
Vif-P1	$A_{151}P$	Hypersensitive	Poor
Vif-Y1	A_{151} Y	Slightly reduced	Normal
Vif-E1	$A_{151}E$	Normal	Normal
Vif-N1	$A_{151}N$	Slightly enhanced	Normal

[a] See Fig. 5.
[b] See Fig. 6.
[c] NA, not applicable.

MATERIALS AND METHODS

Plasmids. The full-length molecular clone pNL4-3 (1) was used for the production of wild-type infectious virus. A Vif-defective variant, pNL4-3vif(−), was constructed by deletion of a *Nde*I/*Pfl*MI fragment in *vif*, resulting in a translational frameshift following amino acid 28 (28). Plasmid pHCMV-G contains the vesicular stomatitis virus glycoprotein G (VSV-G) gene expressed from the immediate-early gene promoter of human cytomegalovirus (62) and was used for the production of VSV-G pseudotypes. For expression of Vif in *trans*, the subgenomic expression vector pNL-A1 (55) was employed. This plasmid expresses all HIV-1 proteins except for *gag* and *pol* products. A Vif-defective variant of pNL-A1, pNL-A1vif(−), was constructed by deletion of an *Nde*I/*Pfl*MI fragment as described for pNL4-3vif(−) (28).

Vif processing site mutants were constructed by two-step PCR. Appropriate primer sets were chosen to PCR amplify the sequence between the *Pfl*MI and *Eco*RI sites of pNL4-3 (nucleotides 5297 and 5743, respectively) using pNL4-3 plasmid DNA as the primary template. Mutagenesis primers were designed to include marker restriction sites for the identification of mutants. PCR fragments were digested with *Pfl*MI and *Eco*RI and cloned into the *Pfl*MI/*Eco*RI sites of pNL-A1. All constructs were verified by sequence analysis. Full-length molecular clones of HIV-1 carrying the various processing site mutants of Vif were constructed by subcloning *Pfl*MI/*Bam*HI fragments from the pNL-A1 constructs into the unique *Pfl*MI/*Bam*HI sites in pNL4-3. Resulting constructs were verified both by restriction analysis and by sequence analysis of the *vif* gene. The characteristics of the individual processing site mutants used in this study are summarized in Table 1.

Antisera. Serum from an HIV-positive patient (AIDS patient serum [APS]) was used to detect HIV-1-specific proteins. The serum does not recognize Vif or Nef and only poorly reacts with gp120 in immunoblot assays. A polyclonal, monospecific antiserum to Vif was raised in rabbits against *Escherichia coli*-derived fusion proteins (28) and was used for all immunoprecipitation and some of the immunoblotting analyses. A monoclonal antibody (MAb) to Vif (MAb 319) was used for some of the immunoblot analyses and was obtained from the NIH AIDS Research and Reference Reagent Program (46, 52).

Tissue culture and transfections. HeLa cells were propagated in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS). H9 and Jurkat T-cell lines were maintained in complete RPMI 1640 medium supplemented with 10% FBS.

For transfection of HeLa cells, cells were grown in 25-cm^2 flasks to about 80% confluency. Cells were transfected using Lipofectamine Plus (Invitrogen Corp., Carlsbad, Calif.) following the manufacturer's recommendations. For Lipofectamine transfections, a total of 4 to 5 μg of plasmid DNA per 25-cm^2 flask was used. Cells were harvested 48 h posttransfection except where indicated otherwise. For transient expression in T-cell lines, cells were electroporated with plasmid DNAs as follows. For each electroporation, 5×10^6 cells were washed once in OptiMEM (Invitrogen Corp.) and suspended in 300 μl of RPMI supplemented with 10 mM D-glucose and 0.1 mM dithiothreitol (DTT). Plasmid DNAs (total of 20 μg per transfection) were diluted into 300 μl of RPMI and combined with the cells. The mixture was then transferred to a sterile electroporation cuvette (Gene Pulser cuvette [0.4 cm]; Bio-Rad) and pulsed with a Bio-Rad Gene Pulser II (975 μF; 0.2 kV). Electroporated cells were subsequently transferred to tissue culture flasks containing 5 ml of RPMI–10% FBS and cultured for 24 to 48 h.

Preparation of virus stocks. Virus stocks were prepared by transfecting HeLa cells with appropriate plasmid DNAs (5 μg/25-cm^2 flask) using Lipofectamine Plus. For the production of VSV-G-pseudotyped virus stocks, HIV plasmids were cotransfected with pHCMV-G (4 μg of viral plasmid DNA plus 1 μg of pHCMV-G plasmid DNA per 25-cm^2 flask). Virus-containing supernatants were harvested 48 h after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 × *g*) and clarified supernatants were filtered (0.45 μm) to remove residual cellular contaminants. Virus-containing supernatants were then concentrated by ultracentrifugation (60 min, 35,000 rpm; SW41 rotor) and suspended in 1 to 2 ml of RPMI. Reverse transcriptase activity was determined on concentrated and unconcentrated virus stocks for virus normalization using a standard reverse transcriptase assay (59).

Metabolic labeling and immunoprecipitation. Transfected HeLa cells (approximately 10^7) were metabolically labeled for 4 h with [^{35}S]methionine (2 mCi/ml; ICN Biomedical, Inc., Costa Mesa, Calif.). Virus produced during the 4-h labeling period was collected, cells were removed by centrifugation, and cell-free virus was divided into four equal aliquots. Virus samples were incubated at 37°C for various times and adjusted to 0.1% Triton X-100 prior to immunoprecipitation. Immunoprecipitations were done as described previously (28) using appropriate antisera as indicated in the text. Proteins were solubilized by boiling in sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Radioactive bands were visualized by autoradiography, and quantitation was performed using a Fuji BAS 2000 Bio-Image analyzer.

In vitro proteolysis of Vif and characterization of processing site. For the production of recombinant Vif in *E. coli*, the *vif* gene from pNL-A1 was amplified by PCR and cloned into the *Bam*HI/*Bgl*II sites of pQE16 (Qiagen) in frame with the six-histidine tag provided by the vector. Recombinant plasmids were used to transform *E. coli* M15[Rep4] cells (Qiagen). Protein expression was induced with isopropyl-1-β-D-galactopyranoside (IPTG) (1 mM) for 4 h at 37°C. Induced bacteria were then pelleted and lysed in lysis buffer (6 M guanidine HCl, 0.1 M sodium phosphate, pH 8.0). Insoluble cell debris was removed by centrifugation at 15,000 × *g* for 30 min at room temperature, and the supernatant was loaded onto an Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen) previously equilibrated with lysis buffer. The column was washed twice with buffer containing 8 M urea and 0.1 M phosphate buffer, pH 6.3. Vif protein was eluted with elution buffer (8 M urea, 0.1 phosphate buffer, pH 4.5) and dialyzed over a 48-h period against 50 mM MOPS (morpholinepropanesulfonic acid)–150 mM sodium chloride containing progressively reduced concentrations of guanidine HCl as reported elsewhere (61). Purified Vif was stored in small aliquots at −70°C.

For in vitro proteolysis, the purified Vif protein (10 μg) was incubated for 1 h at 37°C with HIV-1 Pr (10 μg) in Pr buffer (300 mM sodium acetate, 50 mM EDTA, 50 mM DTT, 0.5 M sodium chloride [pH 4.7]). Proteins were subsequently separated by SDS–15% PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After brief staining with Coomassie brilliant blue R250, bands were cut out and used for automated Edman degradation. Automated Edman sequencing was performed using a Sequenator model Procise 494cLC connected to an online phenylthiohydantoin amino acid analyzer (model 140D; Applied Biosystems). A C_{18} capillary column (250 by 0.8 mm) was used for the separation of the phenylthiohydantoin amino acids. Standard sequencing protocols were used according to the manufacturer's recommendations.

RESULTS

Virion-associated Vif is unstable. While there is general consensus in the literature that Vif can be found in association with viral particles, the amounts of Vif observed in virions are highly variable. One possible explanation for this phenomenon is that virus-associated Vif is subject to proteolytic degradation. To directly address this question we measured the postbudding stability of Vif and other viral components. Metabolically labeled virus was prepared from HeLa cells cotransfected with equal amounts of pNL4-3vif(−) and pNL-A1 plasmid DNAs. This strategy was used previously for efficient expression and packaging of Vif (29). Virus production was allowed for 4 h in the presence of [^{35}S]methionine. Virus-containing supernatants were then cleared by centrifugation to remove cellular contaminants. Equal aliquots of the metabolically labeled virus were incubated at 37°C for various times as indicated in Fig. 1. Individual samples were then adjusted to 0.1% Triton X-100 to disrupt viral membranes and subjected to immunoprecipitation with serum from an HIV-positive patient or a Vif-specific polyclonal antiserum (Fig. 1A). All major viral proteins such as capsid (CA), p41^{gag}, or envelope (Env) were stable over the 19-h observation period (Fig. 1B), the only exception being residual Pr55^{gag} precursor protein that was gradually processed into the mature Gag products. Surprisingly, Vif was also unstable, and only 25% of the Vif originally found at time zero was recovered 19 h later (Fig. 1B). This suggests that virus-associated Vif is subject to postbudding processing or degradation.

Truncated forms of Vif are present in virus preparations from H9 and Jurkat cell lines. To more closely follow the fate of virus-associated Vif, we next tried to identify possible processing or degradation intermediates by using high-percentage gel systems to allow for the identification of small viral proteins. To analyze virus from productively infected cells, H9 cells were infected with the NL4-3 isolate and virus replication was monitored by measuring the reverse transcriptase activities in the culture supernatant at regular intervals (not shown). Virus was harvested on days 8 (passage 2 [P2]), 11 (P3), 15 (P4), and 19 (P5) postinfection. Uninfected H9 cells were added to the culture at each time point to maintain productive infection. Cell-free virus from P4 and P5 and, as a control, culture supernatant from uninfected H9 cells were concentrated by ultracentrifugation and subjected to immunoblot analysis using a Vif-specific MAb (MAb 319) (Fig. 2A). The Vif-specific MAb recognized full-length Vif in the concentrated virus preparations from both passages (Fig. 2A). In addition, several shorter forms of Vif were recognized by the Vif MAb, the most predominant ones having molecular sizes of approximately 7 kDa (p7*) and 17 kDa (p17*), respectively. The same blot was subsequently reacted with serum from an HIV-positive patient (Fig. 2A), confirming the absence of viral proteins in the uninfected culture (Fig. 2A, lane 1) and revealing the presence of equivalent amounts of viral capsid (CA) proteins in P4 and P5 (Fig. 2A, lanes 2 and 3). The results from this experiment demonstrate that virus preparations from productively infected H9 cells indeed contain both full-length and truncated forms of Vif, supporting the notion that Vif is subject to intravirion proteolysis.

We next wanted to determine whether the instability of Vif is producer cell-dependent and denotes a common feature of virus-associated Vif. To accomplish this we studied the steady-state levels of cell- and virus-associated Vif derived from Jurkat and H9 cell lines (Fig. 2B). H9 and Jurkat cells were electroporated with a combination of the Vif-defective NL4-3 molecular clone and either the Vif producer plasmid pNL-A1 (Fig. 2B, lanes 2, 4, 5, 6) or its Vif-defective analog, pNL-A1vif(−) (Fig. 2B, lanes 1 and 3). Cell lysates and concentrated virus preparations were subjected to SDS–15% PAGE, and Vif-specific proteins were identified by immunoblotting using a polyclonal Vif antibody. Full-length Vif was clearly detectable both intracellularly (Fig. 2B, lanes 2 and 5) and in virus preparations (lanes 4 and 6) from Vif-expressing H9 (Fig. 2B, lanes 2 and 4) and Jurkat (Fig. 2B, lanes 5 and 6) cells. No Vif-specific products were detectable in a Vif-negative virus preparation derived from H9 cells (Fig. 2B, lane 3). Intracellularly, a number of Vif-specific products—presumably repre-

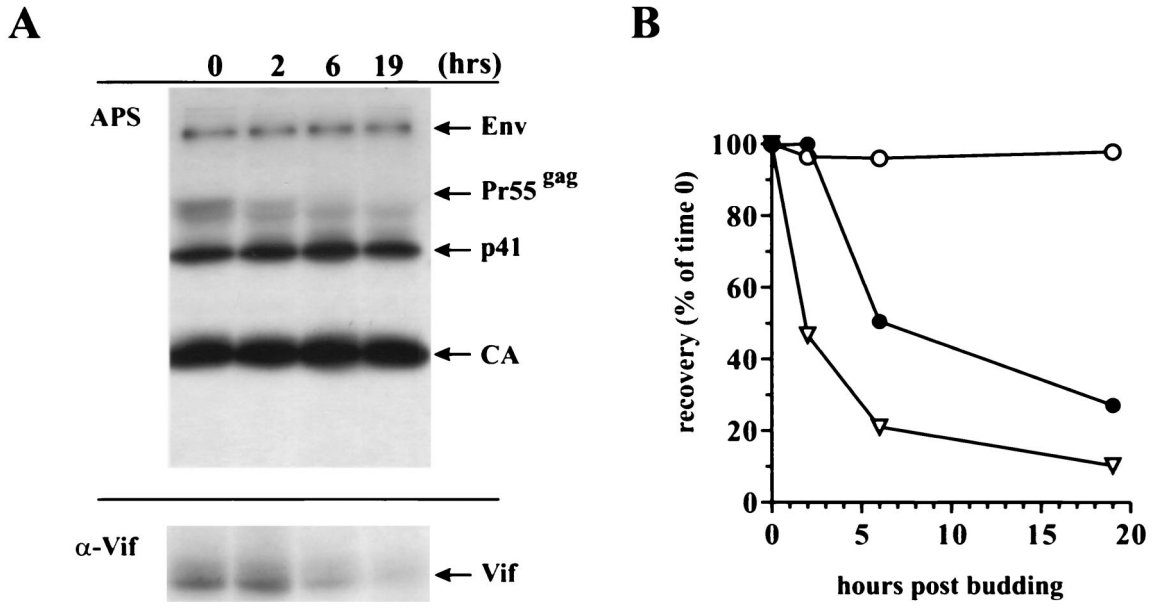

FIG. 1. Virion-associated Vif is metabolically unstable. (A) HeLa cells were cotransfected with equal amounts of pNL4-3vif(−) and pNL-A1 plasmid DNAs. Twenty-four hours later, cells were metabolically labeled for 4 h with [^{35}S]methionine (1 mCi/ml). Virus produced during the 4-h labeling period was harvested, cleared of cellular debris, and separated into four equal fractions. Individual fractions were incubated at 37°C for up to 19 h as indicated above the lanes. Part of each fraction (25%) was then subjected to immunoprecipitation using serum (APS) from an HIV-positive patient. The remaining part (75%) of each fraction was reacted with a Vif-specific polyclonal antiserum. Immunoprecipitated proteins were solubilized by boiling in SB (4% SDS, 125 mM Tris [pH 6.8], 10% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and separated by SDS–14% PAGE. Proteins were visualized by fluorography. (B) The intensities of Vif (●)-, CA (○)-, or Pr55^{gag} (▽)-specific bands were quantified by densitometric scanning. Protein stability was calculated as the percentage of protein recovered relative to time point 0, which was empirically set as 100%.

senting degradation intermediates of Vif—were detectable. Consistent with the results from the productive infection (Fig. 2A), virus preparations from transiently transfected H9 and Jurkat cells revealed several truncated forms of Vif, including the 7-kDa (p7*) and 17-kDa (p17*) products noted above. A product with a size similar to that of p17* was also found in cell lysates, and its presence in virus preparations could therefore reflect copackaging of a truncated cellular form of Vif. In contrast, the p7* product could not be found in cell lysates and was thus produced subsequent to virus release.

Virion-associated Vif is targeted by the viral Pr. Previous reports demonstrated that the HIV-1 accessory protein Nef, which is packaged into virions, is subject to proteolytic processing by the viral Pr (57). To determine whether the Vif-reactive p7* product observed in Fig. 2 is similarly the result of proteolytic processing by the viral Pr, we performed two separate experiments. In the first experiment virions were produced from transiently transfected HeLa cells in either the absence or presence of ritonavir (5 μM), a specific inhibitor of the HIV Pr. Cell lysates and concentrated virus preparations were separated by SDS–15% PAGE and subjected to immunoblotting using a Vif-specific polyclonal antiserum (Fig. 3A) followed by reprobing with serum from an HIV-positive patient (Fig. 3A). As expected, ritonavir treatment efficiently blocked maturation of Gag and Gag-Pol proteins both intracellularly and in the virus pellet, as evidenced by the absence of mature CA and matrix (MA) products in the gel (Fig. 3A, lanes 2 and 4). A band comigrating with MA in the ritonavir-treated cell lysates (Fig. 3A, lane 2) presumably represents Vpu, which comigrates with MA and is recognized by the patient serum used in this study. Truncated forms of Vif, first noted in virus-producing H9 and Jurkat cells (Fig. 2), were clearly detectable in HeLa cells as well. Interestingly, ritonavir treatment had no effect on the appearance of intracellular truncated forms of Vif (Fig. 3A, lanes 1 and 2), indicating that they were not produced by an aberrant, premature intracellular activation of the viral Pr. Instead, they most likely represent degradation intermediates from normal turnover of Vif by cellular Prs. However, ritonavir treatment efficiently blocked the appearance in the viral pellet of the p17* and p7* Vif products present in untreated virus preparations (Fig. 3A, lanes 3 and 4). These results suggest that virus-associated Vif is proteolytically cleaved by the viral Pr subsequent to viral budding. In addition, these data demonstrate that intravirion processing of Vif is producer cell independent, as it was observed in H9, Jurkat, and HeLa cells.

To confirm that intravirion processing of Vif is mediated by the HIV Pr, we performed a second set of experiments that analyzed virion-associated Vif in the context of an HIV variant carrying an active-site mutation in the viral Pr (26). HeLa cells were transfected with wild-type pNL4-3 or pNL4-3 (Pr$^-$) plasmid DNAs (Fig. 3B). Cell lysates and concentrated virus preparations were prepared 48 h later and analyzed by immunoblotting using first a Vif-specific antibody and then serum from an HIV-positive patient (Fig. 3B). Consistent with the results from Fig. 3A, Pr-defective viruses contained significant amounts of full-length Vif (Fig. 3B, lane 8). In addition, several truncated forms of Vif, most of which were also present in cell

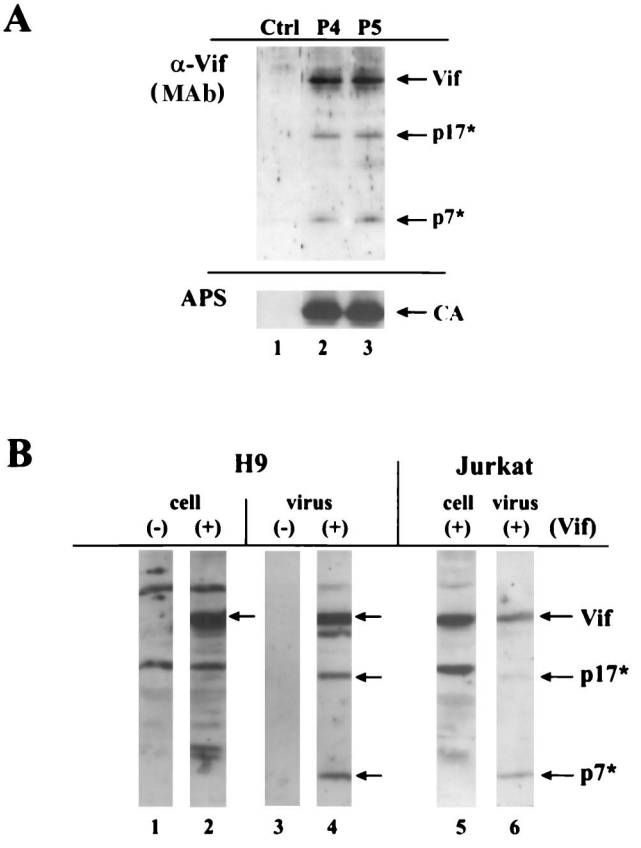

FIG. 2. Proteolytic processing of Vif in virus derived from H9 and Jurkat cells. (A) H9 cells (5×10^6) were infected with a concentrated virus stock of the NL4-3 isolate. Virus production was monitored by reverse transcriptase assay, and virus was harvested near peak infection (day 5). Uninfected H9 cells (5×10^6) were added to the culture to maintain productive infection and virus (P2) was harvested on day 8. The same procedure was repeated to produce virus P3 (day 11), P4 (day 15), and P5 (day 19). Virus derived from P4 and P5 was concentrated by ultracentrifugation. Culture supernatant from uninfected H9 cells was concentrated in parallel (Ctrl). Concentrated samples were separated by SDS–15% PAGE and subjected to immunoblotting using a Vif-specific MAb (MAb 319). The same blot was subsequently reblotted with serum (APS) from an HIV-positive patient. Proteins are identified on the right. (B) H9 or Jurkat cells were transfected by electroporation with a combination of pNL4-3vif(−) and either pNL-A1vif(−) (lanes 1 and 3) or pNL-A1vif(+) (lane 2 and lanes 4 to 6). Cells and viruses were harvested 48 h after electroporation. Cells were pelleted and lysed by boiling in 400 μl of PBS-SB (1:1). Virus-containing supernatants were filtered (pore size, 0.45 μm) to eliminate cellular debris, and virus was pelleted through 20% sucrose. Virus pellets were solubilized in 300 μl of PBS-SB (1:1). Cell lysates (40 μl) and pelleted virus (80 μl) were separated by SDS–15% PAGE, transferred to PVDF membranes, and blotted against a Vif-specific polyclonal antibody. Proteins were visualized by enhanced chemiluminescence (ECL kit; Amersham). Proteins marked by arrows are discussed in the text.

lysates, were identified in these virus preparations. However, no p17* or p7* proteolytic Vif fragments present in wild-type virus (Fig. 3B, lane 7) could be identified (Fig. 3B, lane 8). These results confirm that processing of Vif to produce the p7* product occurs after virus release and requires an active viral Pr. While the release of the p7* fragment from Vif is clearly a postbudding event, the origin and nature of the p17* product is less clear. The sensitivity of the p17* product to ritonavir, visible in Fig. 3A (lane 3), as well as its absence in the Pr-deficient virus preparation (Fig. 3B, lane 7) suggest that it might represent the N-terminal fragment of Vif, derived from intravirion processing, that is recognized by the polyclonal Vif antibody. However, the fact that the Vif MAb used in Fig. 2A similarly recognizes p7* and p17* products argues against such a possibility. In fact, it is possible that the p17* products seen in Fig. 2A and 3 are distinct Vif processing products with similar electrophoretic mobilities. To avoid confusion, we used the appearance of the p7* product as marker for intravirion processing of Vif for all subsequent analyses

Mapping of the Vif processing site. The known processing sites in the HIV-1 Gag and Gag-Pol precursor proteins recognized by the viral Pr are highly diverse. In fact, none of the processing sites are identical. This suggests that the viral Pr recognizes structural motifs on its substrates rather than a linear amino acid sequence. Moreover, our attempts to align Vif sequences to any of the sequences surrounding the known processing sites in Gag and Pol did not reveal any potential proteolytic cleavage sites in Vif (data not shown). This necessitated the characterization of the Vif processing site through experimental methods.

Both the epitope recognized by the Vif MAb used in Fig. 2A and the main epitope recognized by the polyclonal Vif antiserum used in the other experiments map to a region near the C terminus of Vif (unpublished data). The efficient recognition of the p7* fragment by this antibody is therefore an indication that p7* represents a C-terminal processing product. To further map the approximate processing site in Vif, we compared intravirion processing of a series of in-frame deletion mutants mapping to various regions in Vif. The results from such experiments suggested that the processing site in Vif was located downstream of amino acid residue 149 (data not shown). To more precisely determine the processing site in Vif, we developed an in vitro assay using purified recombinant Vif and Pr preparations. Expression and purification of recombinant Vif are described in Materials and Methods. Recombinant HIV-1_{HXB2} KIIA Pr was prepared as previously described (35). For in vitro proteolysis of Vif, 10 μg of purified recombinant Vif was incubated for 1 h at 37°C with KIIA Pr (10 μg) in Pr buffer (300 mM sodium acetate, 50 mM EDTA, 50 mM DTT, 0.5 M sodium chloride [pH 4.7]). Two independent reactions were performed in parallel (Fig. 4A, lanes 2 and 3). Reaction products were separated by SDS–15% PAGE and transferred to PVDF membranes (Immobilon). Unprocessed Vif (Fig. 4A, lane 1) and KIIA Pr (lane 4) were included as controls. Protein bands were visualized by staining with Coomassie brilliant blue R250 (Fig. 4A). In addition to residual unprocessed Vif and the input Pr, two major processing products, P1 and P2, with estimated molecular masses of 17 and 7 kDa, respectively, were identified. The mobilities of P1 and P2 were very similar, if not identical, to those of the p17* and p7* products observed in virus preparation in vivo (Fig. 2A), suggesting that they represent authentic Vif processing products. Areas containing P1 and P2 in lanes 2 and 3 were cut from the filter and used for automated Edman sequencing. Residues identified by sequencing P1 and P2 are shown in Fig. 4B. The results clearly demonstrate that P2 represents the C-terminal fragment of Vif that was derived by processing of Vif following amino acid residue 150.

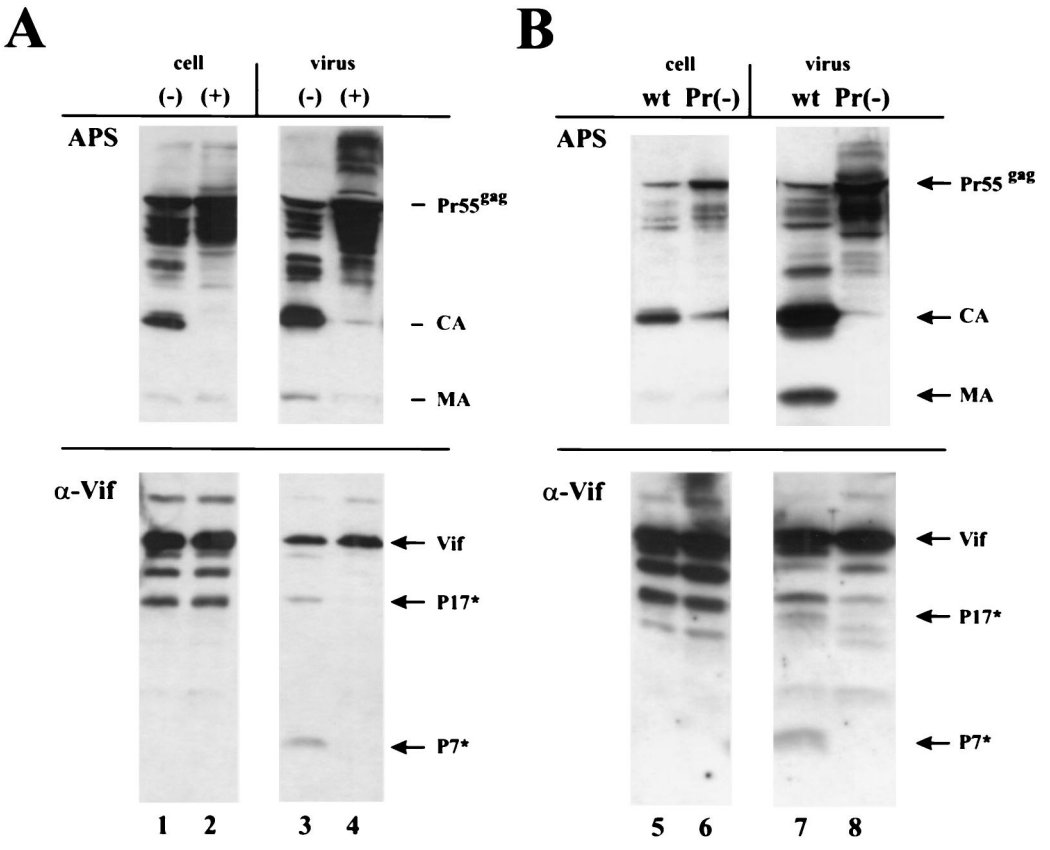

FIG. 3. Intravirion processing of Vif by the viral Pr. (A) Four 25-cm^2 flasks of HeLa cells were cotransfected with pNL4-3vif(−) and pNL-A1 plasmid DNAs. Two flasks were treated with ritonavir (5 μM) immediately after transfection (lanes 2 and 4), while the other two flasks were left untreated (lanes 1 and 3). Cells and virus-containing supernatants were harvested 48 h following transfection; duplicate flasks were pooled and processed for immunoblotting as described in the legend to Fig. 2. Cell lysates and concentrated viral pellets were separated by SDS–15% PAGE and blotted against serum (APS) from an HIV-positive patient or a Vif-specific polyclonal antiserum (α-Vif). Proteins are identified on the right. The presence (+) or absence (−) of ritonavir is indicated above the lanes. (B) HeLa cells were transfected with pNL4-3 or pNL4-3(Pr$^-$) plasmid DNAs per flask. Cells and virus-containing supernatants were harvested and processed as described for panel A.

Vif variants from various isolates have been previously shown to be capable of functional complementation despite a considerable variability in their primary sequences (41, 50). To get an appreciation for the potential significance of the proteolytic processing of Vif in mature virions, we compared the Vif sequences of a number of HIV-1, HIV-2, and SIV isolates (Fig. 4C). The alignment of 12 Vif sequences representing four variants each of HIV-1, HIV-2, and SIV demonstrates significant variability in the Vif primary sequence. However, it is also apparent that Vif contains a number of conserved domains; notably, a stretch of 7 amino acids corresponding to residues 144 to 150 in the NL4-3 isolate was found to be highly conserved among all HIV-1, HIV-2, and SIV isolates analyzed. Interestingly, the Vif processing site is located immediately adjacent to this highly conserved domain. This suggests that the proteolytic processing site in Vif is conserved not only in HIV-1 but also in HIV-2 and SIV. Thus, it is possible that intravirion processing is not restricted to HIV-1 Vif isolates but could occur in HIV-2 or SIV Vif as well.

Mutations at the cleavage site affect intravirion processing of Vif. To address the importance of intravirion processing of Vif for its biological activity, we constructed a series of mutations at or near the processing site that introduced single or triple amino acid substitutions into the Vif sequence. The goal was to change the structure of the Vif processing site by replacing the nonpolar residues surrounding the Vif processing site with charged residues or with proline because of its effect on protein structure. All mutants were created by two-step PCR mutagenesis as described in Materials and Methods. A summary of the mutants analyzed in this study is listed in Table 1. Expression and packaging of the individual Vif variants expressed in HeLa cells from pNL-A1 in the presence of the Vif-defective pNL4-3vif(−) vector were analyzed by immunoblotting of cell lysates or concentrated virus preparations (Fig. 5). Intracellular expression levels were very similar for all mutants (Fig. 5), and all proteins exhibited similar intracellular processing and degradation profiles. The Vif-RKS mutant (Fig. 5, lanes 2 and 8) exhibited a slightly slower mobility than wild-type Vif (Fig. 5, lanes 1 and 7) presumably due to the presence of two extra positively charged amino acid residues in this mutant. All Vif variants were packaged with similar efficiency as wild-type Vif (Fig. 5, lanes 7 to 12). However, intravirion processing of Vif, as measured by the appearance of the p7* product, was significantly different. Vif-RKS (Fig. 5, lane 8) was almost completely resistant to processing by the viral Pr, while Vif-P1 (Fig. 5, lane 10) was hypersensitive. One process-

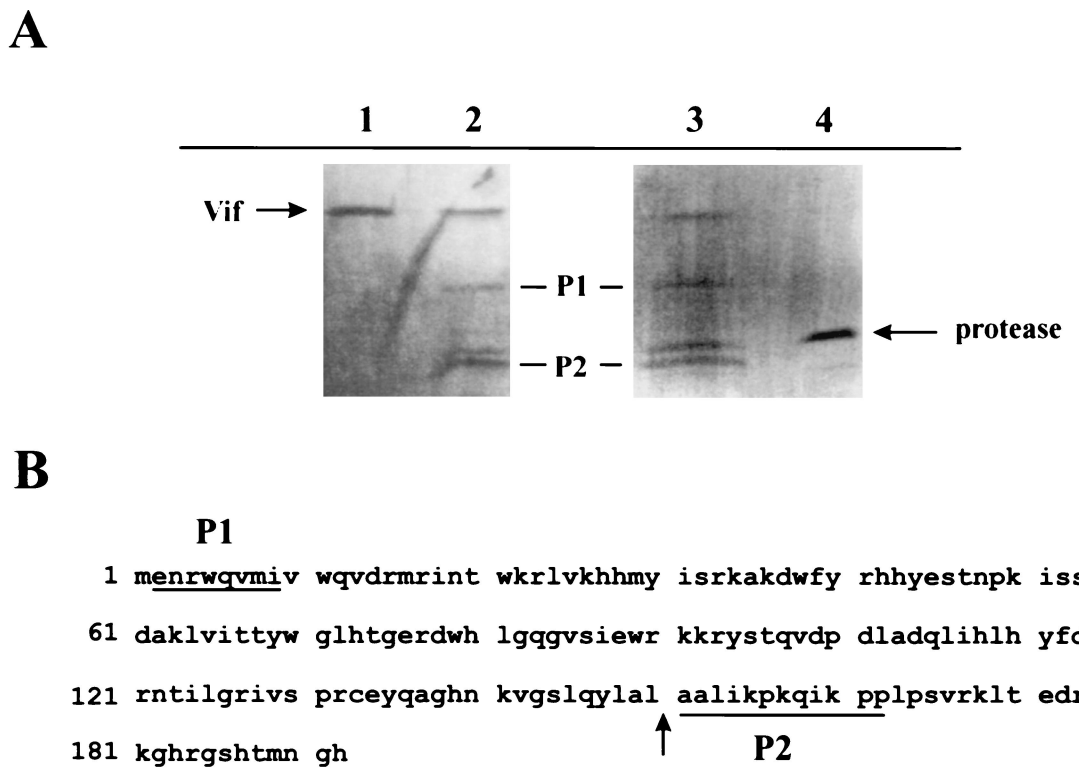

FIG. 4. In vitro processing of Vif and identification of the Vif processing site. (A) Recombinant Vif (10 μg) was incubated with KIIA Pr (10 μg) for 1 h at 37°C in Pr buffer (300 mM sodium acetate, 50 mM EDTA, 50 mM DTT, 0.5 M sodium chloride [pH 4.7]). Proteins were then separated by SDS–15% PAGE, transferred to PVDF membrane, and visualized by brief staining with Coomassie brilliant blue R250. Untreated recombinant Vif (lane 1) and purified Pr (lane 4) were included for reference. Protein bands labeled as P1 and P2 (lanes 2 and 3) were cut from the membrane and used for sequence analysis as described in Materials and Methods. (B) The predicted amino acid sequence of Vif is shown. Sequences verified by peptide sequencing of P1 and P2 fragments are underlined. The arrow marks the Vif processing site. (C) Alignment of four representative Vif sequences each of HIV-1, HIV-2, and SIV. Highlighted blocks denote sequence identity of more than 60% of the aligned sequences. The arrow denotes the Vif processing site. A highly conserved sequence immediately upstream of the processing site is underlined.

ing-site mutant, Vif-E1 (Fig. 5, lane 9), exhibited similar sensitivity to processing as wild-type Vif, while mutants Vif-Y1 and Vif-N1 (Fig. 5, lanes 11 and 12, respectively) had slightly reduced or increased sensitivity to processing, respectively. It is important to point out that all mutants, except for Vif-RKS, contained a single amino acid change at residue 151 immediately downstream of the processing site. Thus, mutation at position 151 in Vif had differential effects on Vif processing depending on the nature of the substitution. Vif-RKS carries a mutation at residue 151 ($A_{151}S$) but, unlike the other mutants, has two additional changes at residues 149 and 150 (AL to RK) in the conserved domain of Vif. The significantly increased resistance of this mutant to processing could suggest a role of the conserved domain in the processing of Vif by the viral Pr. Taken together, our results demonstrate that mutation of the Vif processing site can either increase or decrease the sensitivity of Vif to proteolytic processing by the viral Pr depending on the amino acid substitution.

Replication of Vif processing variants in restrictive or permissive target cells. To directly address the role of Vif processing on its ability to support virus replication in restrictive target cells, Vif processing site mutants were transferred into the infectious molecular clone NL4-3. Virus stocks were prepared in HeLa cells, normalized for equal RT activities, and used for the infection of Jurkat and H9 cells (Fig. 6). Jurkat cells exhibit a fully permissive phenotype and do not restrict replication of Vif-defective variants. In contrast, H9 cells are highly restrictive, and HIV replication in these cells is critically dependent on Vif function. To ensure equivalent levels of primary infection of these cells, virus stocks were pseudotyped with VSV-G. Virus replication was monitored over a 10-day period, and aliquots of the culture supernatants were harvested in 2-day intervals and analyzed for virus content using a standard reverse transcriptase assay (59). As expected, all processing site mutants had replication profiles in Jurkat cells that were indistinguishable from those of wild-type or Vif-defective viruses (Fig. 6). In contrast, only three of the processing-site mutants, Vif-E1, Vif-Y1, and Vif-N1, were capable of supporting efficient virus replication in H9 cells (Fig. 6). Interestingly, Vif-E1, Vif-Y1, and Vif-N1 are the mutants whose processing profile was closest to that of wild-type Vif. Vif-P1, which exhibited significantly increased sensitivity to processing, was severely impaired in its activity but supported a residual low level of virus replication in H9 cells. In contrast, Vif-RKS, which was completely resistant to processing by the viral Pr, was incapable of supporting virus replication in H9 cells. These results demonstrate that mutations in Vif that induce a profound change in the sensitivity to Pr-dependent processing also severely affected Vif function suggesting that intravirion processing of Vif is important for the production of infectious viruses.

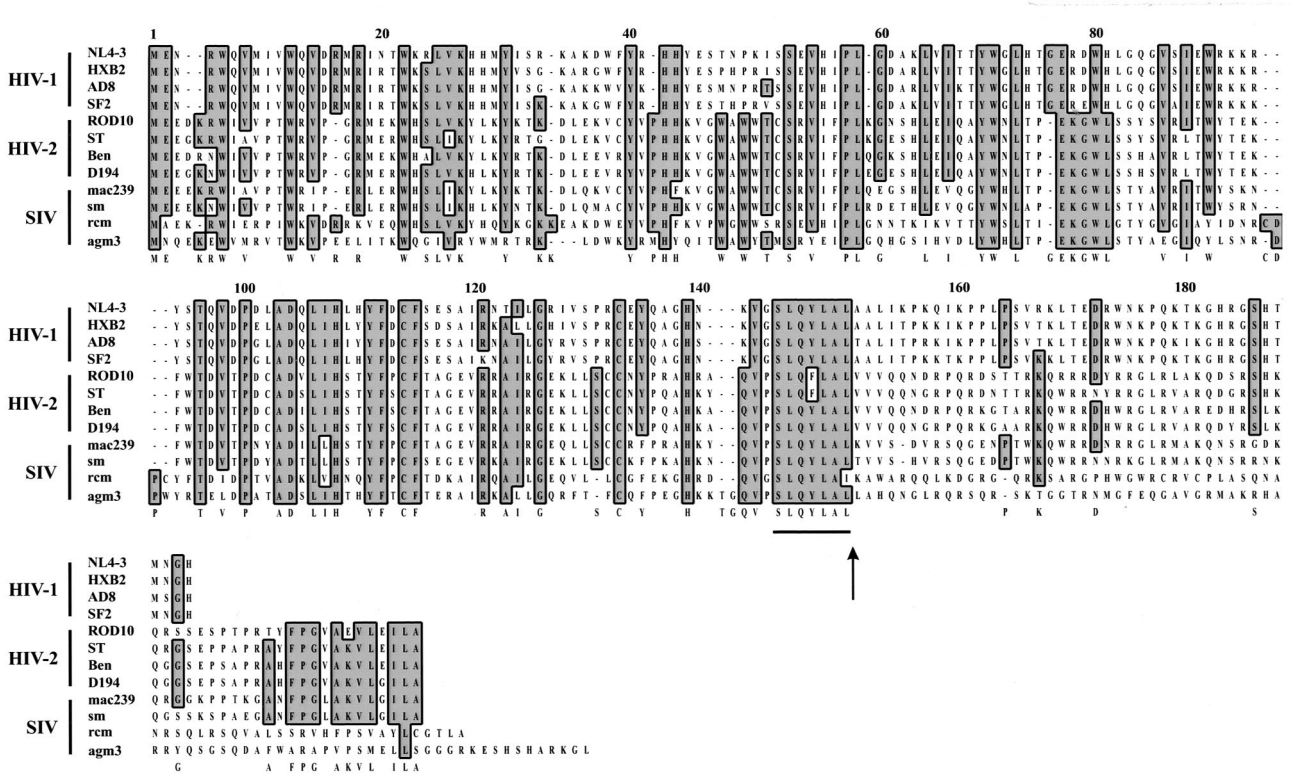

FIG. 4—*Continued.*

DISCUSSION

While the importance of Vif for viral infectivity in primary human cells and some transformed cell lines is undisputed, the question of how Vif might accomplish this task is still under debate. In particular the site of Vif function, i.e., intracellular or viral, remains under investigation. We and others have previously noted that the amount of Vif packaged into progeny virions is proportional to the intracellular expression levels (29, 49) corresponding to approximately 10 to 15% of total Vif protein (29). The packaging efficiency of Vif is therefore comparable to that of Env (58). In addition, the results from our study offer a possible explanation for the apparent paucity of virus-associated Vif reported in previous studies (14, 54), which could be accounted for by the gradual processing of full-length virus-associated Vif into a short C-terminal fragment (p7*), carrying the major antigenic epitope, and a longer N-terminal fragment that is generally not or only very inefficiently recognized by available polyclonal antibodies or MAbs (not shown). Thus, our results suggest that packaging of Vif occurs at levels that match or exceed those of reverse transcriptase or integrase, supporting the notion that packaging of Vif might be functionally relevant.

On the other hand, results from heterokaryon analyses suggested that Vif acts to neutralize an endogenous inhibitor of HIV replication, thus favoring an intracellular function of Vif (34, 47). While the nature of the proposed cellular inhibitor remains elusive, a number of cellular Vif-interacting proteins have been identified to date, including vimentin or a vimentin-associated factor (24, 28), HP68 (64), or Hck (23). Among those, only Hck was reported to interfere with virus replication in a Vif-dependent manner (23). In addition, Vif was reported to interact with Gag proteins (5, 8, 27) as well as viral or cellular RNA (13, 63), suggesting that Vif has multiple functional domains to interact with a variety of cellular or viral factors. In fact, it seems possible that Vif, similar to Vpr, Nef, or Vpu, acts as a molecular adapter to connect cellular and/or viral factors. If and which of these interactions are functionally relevant remains to be shown. Given the comparatively low levels of Vif packaged into virus particles, a virus-associated function of Vif would require a mechanism relying on only small quantities of Vif for full activity. Indeed, it is not difficult to envision scenarios that would fulfill this requirement. For example, it is possible that Vif functions as a cofactor to stabilize components of the viral reverse transcription complex. Such a function would be consistent with the reported association of Vif with nucleoprotein complexes (28, 29, 32) as well as the effect of Vif on the stability of nucleoprotein or reverse transcription complexes (15, 25, 39, 48) and would require levels of Vif comparable to those of reverse transcriptase or integrase. There are other possible scenarios involving virus-associated Vif, such as the temporal modulation of the viral Pr activity during virus maturation (27, 31, 40). Such a function of Vif would be consistent with the reported inhibition of Gag processing by Vif or Vif-derived peptides in vitro (4, 19, 31). However, such Vif-dependent modulation of Gag processing has thus far not been consistently observed in vivo, and future

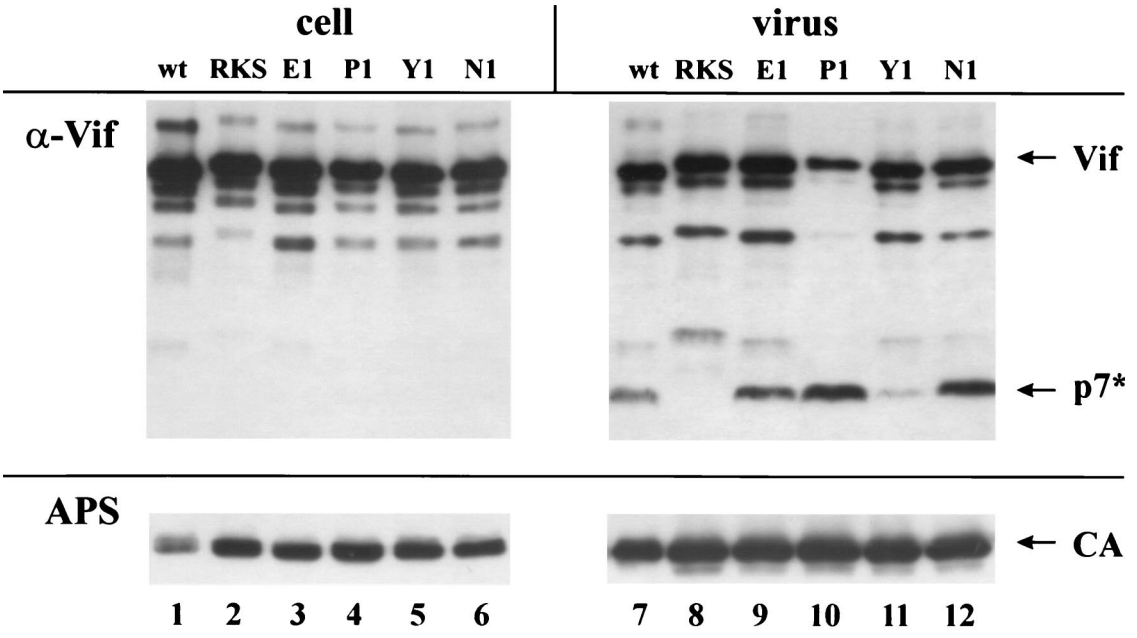

FIG. 5. Biochemical characterization of Vif processing site mutants. HeLa cells were transfected with a combination of pNL4-3vif(−) and any of the pNL-A1-based Vif expression vectors as indicated above the lanes. Cells and virus-containing supernatants were harvested 48 h following transfection and processed for immunoblotting as described in the legend to Fig. 2. Cell lysates and concentrated viral pellets were subjected to SDS–15% PAGE. Proteins were transferred to PVDF membranes and subjected to immunoblotting using a Vif-specific antiserum (α-Vif). Proteins were visualized by enhanced chemiluminescence. The same blots were then stripped and reacted with serum (APS) from an HIV-positive patient to control for comparable levels of virus production. Proteins are identified on the right.

experiments will have to show its possible correlation with Vif-dependent regulation of viral infectivity in vivo.

Additional evidence for a virus-associated function of Vif comes from our observation that Pr-dependent processing of Vif is restricted to cell-free virus. It is interesting that replacement of Ala_{151} by Glu, Pro, Tyr, or Asn had different effects on Vif processing, ranging from inhibition of processing to facilitation of Pr-dependent proteolysis (Fig. 5). These differential effects on Vif processing are best explained by structural changes in Vif at or near the processing site that either impair or facilitate access by the viral Pr. While it cannot be ruled out that such structural changes in the Vif protein are directly responsible for the observed effects on Vif function, the parallels between alterations in the sensitivity of individual Vif variants to Pr-dependent proteolysis and Vif function present compelling, albeit indirect, evidence to suggest a correlation between Vif-processing and its regulation of viral infectivity. The potential functional significance of Vif is further highlighted by the fact that Vif processing occurs immediately downstream of a domain that is highly conserved not only between HIV-1 but also HIV-2 and SIV isolates (Fig. 4C). This could suggest that the processing site is conserved among lentivirus Vif proteins. In fact, preliminary experimental evidence suggests that both HIV-2 and SIV Vif proteins are subject to intravirion processing by HIV-2 and SIV Pr, respectively. Thus, packaging and intravirion processing of Vif is not a phenomenon limited to individual HIV-1 isolates but appears to be a general feature of Vif proteins in primate immunodeficiency viruses (unpublished data).

It is currently unclear exactly which residues constitute the recognition motif for the viral Pr in Vif. Mutations at Ala_{151} were found to be either neutral or to increase or decrease sensitivity to proteolytic processing, depending on the nature of the amino acid substitution (Fig. 5). In contrast, substitution of the highly conserved Ala_{149}/Leu_{150} residues with two positively charged residues (Arg_{149}/Lys_{150}) in the Vif-RKS mutant completely abolished Pr-dependent processing of Vif. Interestingly, deletion of amino acid residues 144 to 149, which includes the conserved SLQYL motif, did not affect Vif processing (data not shown) yet abolished Vif function (51; our unpublished data). These results suggest that the conserved SLQYL motif in Vif is not primarily a recognition motif for the viral Pr but plays a different, thus far unknown, role in Vif activity. Our results further support the notion that the Vif processing site is defined by structural constraints rather than by a specific amino acid sequence.

It is currently unclear how processing of Vif could be involved in regulating viral infectivity. One possible benefit of Vif processing could be the elimination of the C-terminal domain in Vif, which appears to be important for the interaction of Vif with a variety of cellular and viral factors (8, 21, 22, 53) and may be involved in Vif multimerization (60). It is possible that processing of Vif serves the purpose of abolishing interactions between viral or host factors bound to N- or C-terminal domains of Vif, respectively. In fact, deletion of the C-terminal 22 amino acids was found to abolish Vif interaction with $Pr55^{gag}$ (8), while smaller deletions at the C terminus did not appear to affect Vif function (37, 43). Such interactions may be important during virus assembly but could become inhibitory to final maturation events subsequent to virus release. Alternatively, processing of Vif might induce a conformational change that could either create or mask possible functional domains, such

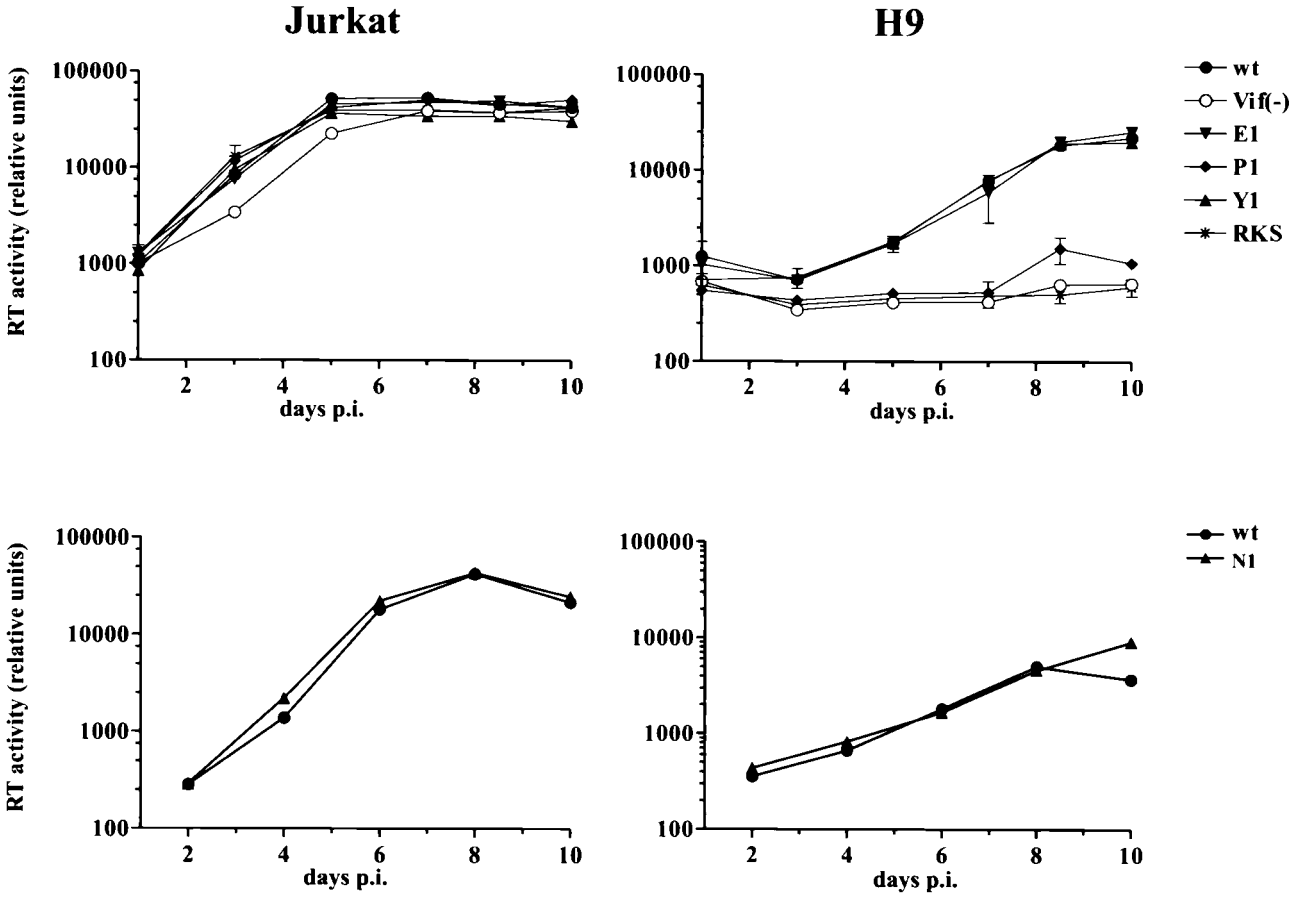

FIG. 6. Replication of NL4-3 variants encoding Vif processing site mutants in Jurkat and H9 cells. Virus stocks for the infection of permissive Jurkat or restrictive H9 cell lines were prepared by transfecting HeLa cells with pNL4-3-based variants of the processing mutants analyzed in Fig. 5. Wild-type NL4-3 and Vif-defective NL4-3vif(−) were included as controls. To ensure efficient initial infection of the target cells, virus stocks were pseudotyped with VSV-G as described in Materials and Methods. Cells were infected, and culture supernatants were collected at 2-day intervals starting on day 1 for 10 days. Virus replication was monitored by determining the reverse transcriptase activity in the culture supernatants using a standard assay (59). Reverse transcriptase activity was plotted as a function of time.

as the conserved SLQYL motif in the N-terminal portion of Vif. Modulation of protein function by proteolytic processing is a common mechanism observed in a variety of biological processes. For instance, many secreted enzymes are synthesized in an inactive form to protect the producer cell from damage by intracellular enzyme activity. In the case of Vif, premature processing could lead to aberrant intracellular distribution or abnormal function. Preliminary experiments involving truncated forms of Vif indeed suggest that processed Vif has biophysical properties that are quite distinct from those of full-length Vif (unpublished data). The extent to which these differences are relevant to Vif function is under investigation.

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